REMARKS

Applicant has rectified minor deficiencies in claims 1 and 18. Claims 1-21 are currently pending. Reconsideration of the application, as amended, is requested in view of the remarks below.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 1-17 are rejected as failing to comply with the enablement requirement. See the Office Action, page 2, lines 5-8.

Independent claim 1 covers a method of inhibiting the growth of tumor cells in a tumor site in a subject by administering to the tumor site an effective amount of an oligoaniline and subsequently exposing the tumor site to irradiation. The oligoanilines covered by formula (I) recited in claim 1 contain at least one hydrophilic group. These oligoanilines possess two common structural features: (1) capability of generating free radicals upon irradiation (due to the presence of electron-rich oligoaniline moieties) and (2) enhanced water-solubility (due to the presence of hydrophilic groups) and, thus, high bioavailability[1]. Also see the Specification, page 1, lines 8-10 and page 3, lines 21-24. To practice the method of claim 1, one first delivers an oligoaniline of formula (I) to a tumor site **only** and then irradiate the site to generate free radicals. The free radicals convert surrounding molecular oxygen to highly reactive oxygen radicals, which in turn attack and damage tumor cells, thereby inhibiting their growth. See, e.g., the Specification, page 1, lines 8-10 and page 3, lines 21-24. In short, given the two common structural features mentioned above, all of the oligoanilines of formula (I) can be used to inhibit the growth of tumor cells in a tumor site by the above-described photodynamic method.

According to the Examiner, "even though claimed breadth of compounds are capable [of] generat[ing] free radicals due to oligoaniline moieties, the encompassed compounds are different from each other in their structure that one of an ordinary skill in the art would have to perform undue experimentation to test each of the compounds that fall in this category in their ability to

[1] The term "bioavailability" refers to the degree to which a drug becomes available to a target tissue after administration. A drug having a higher water solubility can be readily dissolved in water for administration and also has a higher degree of availability to a target tissue.

bind all kinds of tumor cells, generate free radicals to the extent that all kinds of tumor cells or all types or degrees of tumors are effectively inhibited." See the Office Action, page 2, line 19 to page 3, line 2. Applicant disagrees.

As correctly pointed out by the Examiner, the oligoanilines covered by formula (I) recited in claim 1 are different from each other. However, they all share the two above-mentioned common structural features and, due to the two structural features, they all can exert inhibition activity on tumor growth. It is a mere routine procedure to determine the degrees of therapeutic efficacy among different oligoanilines having the two common structural features.

Further, contrary to the Examiner's assertion, enablement of claim 1 does not require testing the efficacy of each oligoaniline of formula (I) recited therein. The law does not impose such a formidable burden on inventors seeking patent protection. "Appellants (here, Applicants) are **not** required to disclose every species encompassed by their claims even in an **unpredictable** art" (emphases added). *In re Angstadt*, 190 USPQ 214, 218 (CCPA 1976). Such a holding is only reasonable, since it is very difficult, if not impossible, to test and disclose all operative species in the chemical and biotechnology fields. Indeed, narrowing the scope claim 1 is unreasonable as it would lead to a grossly unjust result: other persons of ordinary skill in the art could easily avoid the claimed invention by conveniently selecting and using a untested embodiment, without undue experimentation.

The *Angstadt* court stated that, "[w]ithout undue experimentation or effort or expense the combinations which do not work will readily be discovered and, of course, nobody will use them and the claims do no cover them." *Id*, at 219. Admittedly, a considerable, but not undue, amount of experimentation is required to identify more efficacious oligoaniline compounds. However, as pointed out in the response dated June 3, 2004, it is well established that "[a] considerable amount of experimentation is permissible, if it is merely routine, ..." In re Wands, 8 USPQ2d 1400, 1404 (CAFC 1988), citing In re Jackson, 217 USPQ 804, 807 (CCPA 1969). Since the practice of the method of claim 1 is routine, the amount of the experimentation to identify more efficacious oligoanilines is permissible even though it is considerable.

Applicants would like to further point out that, contrary to the Examiner's assertion, enablement of claim 1 also does not require testing the efficacy of an oligoaniline in all types of tumors. All that is required is to determine the degree of its efficacy in inhibiting cell growth for

tumors having a tumor site. Note that the method of claim 1 involves inhibiting the growth of tumor cells by attacking them with free radicals. It involves a photodynamic process applicable to all types of tumors having a tumor site, not a specific biological process applicable only to a certain type of tumors. One skilled in the art would know how to determine the degree of an oligoaniline's efficacy in inhibiting the cell growth of tumors have a tumor site. Indeed, it is a mere routine procedure. In view of the laws set forth by the *Angstadt* and *Wands* courts, enablement of claim 1 does not require testing all types of tumors.

The Examiner also contends that "applicant[] ha[s] not established a rationale to show to one of [] ordinary skill in the art that claimed oligoanilines could generate oxygen free radicals specifically in tumor cells and not in [] normal cells surrounding the tumor cells." See the Office Action, page 3, lines 2-5.

Applicant submits that a skilled person would readily understand the rationale in view of the Specification and common knowledge in the art. First, an oligoaniline covered by formula (I) recited in claim 1 is delivered to a tumor site **only**. See, e.g., Example 4 of the Specification. Second, it is well known that tumor tissues differ substantially from normal tissues. For example, unlike blood vessels in normal tissues, tumor blood vessels are irregularly spaced and highly permeable to plasma. See the highlighted portion in Dvorak, *American Journal of Pathology*, 2003, 162:1747-1757, a copy of which is attached hereto as "Exhibit A." Thus, an oligoaniline covered by formula (I) recited in claim 1 predominantly enters tumor tissues, rather than normal tissues, through plasma. Further, when practicing the method of claim 1, only tumor tissues, but not normal tissues, are irradiated. See, e.g., the Specification, page 5, lines 18-24 and page 9, lines 2-6. Even if an oligoaniline reaches normal tissues, it would not generate free radicals in the absence of irradiation. Thus, one skilled in the art would know that, upon irradiation, an oligoaniline used in the method of claim 1 would generate free radicals predominantly in tumor tissues rather than in normal tissues. The above rationale is adequately supported by the data shown in the Specification. In particular, Example 4 in the Specification describes that the average tumor weight of the mice treated with an oligoaniline of formula (I) was about 40% (a 60% reduction) of the average tumor weight of the untreated mice.

Further, the Examiner asserts that "[o]ne of [] ordinary skill in the art would not readily envision the method of inhibiting tumor cells that are metastatic." See the Office Action, page 3, lines 5-6.

Applicant would like to point out that claim 1 covers a method of inhibiting the growth of tumor cells in a tumor site, regardless of whether the tumor cells are metastatic or not. Of note, claim 1 does **not** cover a method of **curing** all types of tumors. It only covers a method of inhibiting tumor cell growth in a tumor site. Given the non-specific nature of photodynamic therapy (see discussion above), one skilled in the art could envision that the oligoanilines covered by formula (I) recited in claim 1 can be used to inhibit the cell growth of all types of tumor (including metastatic tumor) as long as the cells are in a tumor site.

Finally, the Examiner points out that "[w]ith respect to the argument regarding the *in vitro* and *in vivo* data, applicants have not shown if murine sarcoma cells are a prototype and representative of all types of tumors known-to-date. Reasonable correlation must exist between the scope of the claims and scope of enablement set forth." See the Office Action, page 3, lines 6-9.

As mentioned above, claim 1 only covers a method of inhibiting the growth of tumor cells as long as they are in a tumor site. Sarcoma is a solid tumor arising from connective tissues. It represents all types of solid tumors having a tumor site, where the tumor cells can be attacked by the free radicals generated by an oligoaniline covered by formula (I) recited in claim 1 upon irradiation. Again, inhibiting tumor cell growth by free radicals, as discussed above, is a photodynamic process applicable to all types of tumors having a tumor site, not a specific biological process applicable only to a certain type of tumors. One skilled person in the art can reasonably expect that, if an oligoaniline can be used to inhibit the growth of sarcoma cells, it most likely can also be used to inhibit the growth other tumor cells in a tumor site. Thus, Applicant submits that a reasonable correlation exists between the scope of claim 1 and its enablement from the art and from the Specification.

For the reasons set forth above, claim 1, as well as claims 2-16 dependent from it, is adequately enabled. **It is submitted that claims 1-17, not subjected to other grounds of rejection, are now in condition for allowance.**

Rejection under 35 U.S.C. § 103(a)

Claims 18-21 are rejected as being obvious over Nguyen et al., Macromolecules, 1994, 27, 3625-3631 ("Nguyen"). See the Office Action, page 3, lines 15-16.

Claim 18 covers a pharmaceutical composition containing an oligoaniline of formula (I) and a pharmaceutically acceptable carrier.

Nguyen discloses synthesis and properties of certain water-soluble conducting polyaniline copolymers. It does not disclose or suggest a pharmaceutical composition containing both a polyaniline copolymer and a pharmaceutically acceptable carrier, as required by claim 18.

The Examiner contends that Nguyen discloses solubilizing polyaniline polymers in dispersants (see page 3626, column 1, first paragraph) and that these dispersants are equivalent to pharmaceutically acceptable carriers. Applicant disagrees. Nguyen describes physical properties (e.g., conductivities and color changes) of certain water-soluble conducting polyaniline copolymers. Nowhere in Nguyen is a composition containing a polyaniline polymer and a pharmaceutically acceptable carrier disclosed or even suggested. The pharmaceutical compositions of claim 18 are developed based on the following facts: (1) blood vessels in tumor tissues are much more permeable to plasma than those in normal tissues, (2) cell growth can be inhibited by free radicals, (3) the oligoanilines used in the compositions of claim 18 are capable of generating free radicals upon irradiation, and (4) these oligoanilines possess enhanced bioavailability and are suitable for use in a pharmaceutical composition. Nguyen does not disclose or suggest any of these facts. Thus, one skilled in the art, in view of Nguyen, would not have been motivated to use polyanilines in the pharmaceutical compositions of claim 18.

For the reasons set forth above, claim 18 is not rendered obvious by Nguyen. Neither are claims 19-21, all of which depend from claim 18.

CONCLUSION

Applicant submits that the grounds for rejection asserted by the Examiner have been overcome, and that claims 1-21, as pending, define subject matter that is enabled and nonobvious. On this basis, it is submitted that all claims are now in condition for allowance, an action of which is requested.

Enclosed is a check for the Petition for Extension of Time fee. Please apply any other charges to deposit account 06-1050, referencing Attorney's Docket No.: 06897-006001.

Respectfully submitted,

Date: 3-22-05

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blood vessel leakage (means permeability)

(*American Journal of Pathology.* 2003;162:1747-1757.)

Rous-Whipple Award Lecture

How Tumors Make Bad Blood Vessels and Stroma

Harold F. Dvorak
From the Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts

Solid tumors are composed of two distinct but interdependent compartments: the malignant cells themselves (parenchyma) and the supporting connective tissue (stroma) that they induce and in which they are dispersed.[1,2] The quantity of stroma is highly variable from tumor to tumor but all solid tumors, regardless of type or cellular origin, require stroma for nutrition and waste disposal if they are to grow beyond minimal size.[3]

The discrete separation of parenchymal cells from stroma is not unique to tumors. Normal tissues are also comprised of avascular parenchyma that abuts on vascularized connective tissue stroma. However, tumor stroma differs strikingly from normal connective tissue. Blood vessels offer one example. Tumor vessels differ from their normal counterparts with respect to organization, structure and function.[2,4-7] Unlike the normal vasculature, tumor vessels are not arranged in a hierarchical pattern but are instead irregularly spaced and structurally heterogeneous. They are also hyperpermeable to plasma and plasma proteins, may lack pericytes, and are lined by actively dividing endothelial cells. Other elements of tumor stroma are also abnormal: increased amounts of plasma protein-rich interstitial fluid; structural proteins not normally found in mature stroma such as fibrin, tenascin, and fetal forms of fibronectin; abnormal proteoglycans; variable numbers of inflammatory cells, etc.

Given these extensive differences in composition, it seemed likely to us that tumor and normal stroma were formed by different steps and mechanisms. Although normal stroma generation is not completely understood, it is known to require the cooperative activities of many different cytokines and inhibitors, each expressed in appropriate amounts and temporal sequence.[8-10] Among these are vascular permeability factor/vascular endothelial growth factor (VPF/VEGF, VEGF-A), other members of the VPF/VEGF family such as placenta growth factor (PlGF), and other cytokines such as TGF-ß, PDGF, ephrins, FGFs, angiopoetins-1 and -2, and their respective receptors and inhibitors. By contrast, the imperfect nature of tumor stroma suggested to us that it was generated by a simpler process, one that deviated from normal developmental stromagenesis by having fewer steps and involving fewer cytokines.

The work reported here summarizes our progress over the past 25 years toward elucidating the steps and mechanisms of tumor angiogenesis and stromagenesis. A major conclusion is that a single cytokine, the 164/5 amino acid splicing variant of VEGF-A, is capable of inducing the formation of tumor-like blood vessels and connective tissue stroma. Further, VEGF-$A^{164/5}$ is expressed not only by malignant tumors but also in healing wounds, in cellular immunity, and in chronic inflammatory diseases such as psoriasis and rheumatoid arthritis.[4,5] Therefore, VEGF-$A^{164/5}$ provides a unifying principle that contributes importantly to many, if not all, examples of pathological angiogenesis and tissue repair.

Discovery of Vascular Permeability Factor/Vascular Endothelial Growth Factor (VPF, VPF/VEGF, VEGF-A)

VEGF-A was discovered as the result of microscopic studies of transplantable tumors at early stages after their implantation into syngeneic animals.[11,12] The critical observation was that tumors began as clumps of neoplastic cells dispersed in a fibrin gel stroma (Figure 1, a–d)◆ . Fibrin results from the

clotting of plasma fibrinogen. Fibrinogen, like other plasma proteins, is normally retained within blood vessels; therefore, the presence of extravascular fibrin deposits strongly implied that tumor blood vessels were hyperpermeable to fibrinogen and presumably therefore to other plasma proteins as well. To test this hypothesis, we injected various macromolecular tracers intravenously into tumor-bearing animals and found extensive tracer extravasation from tumor as compared with normal blood vessels (Figure 1, e and f)✦ . Several earlier papers had also commented on the increased permeability of tumor blood vessels (reviewed in[5]).

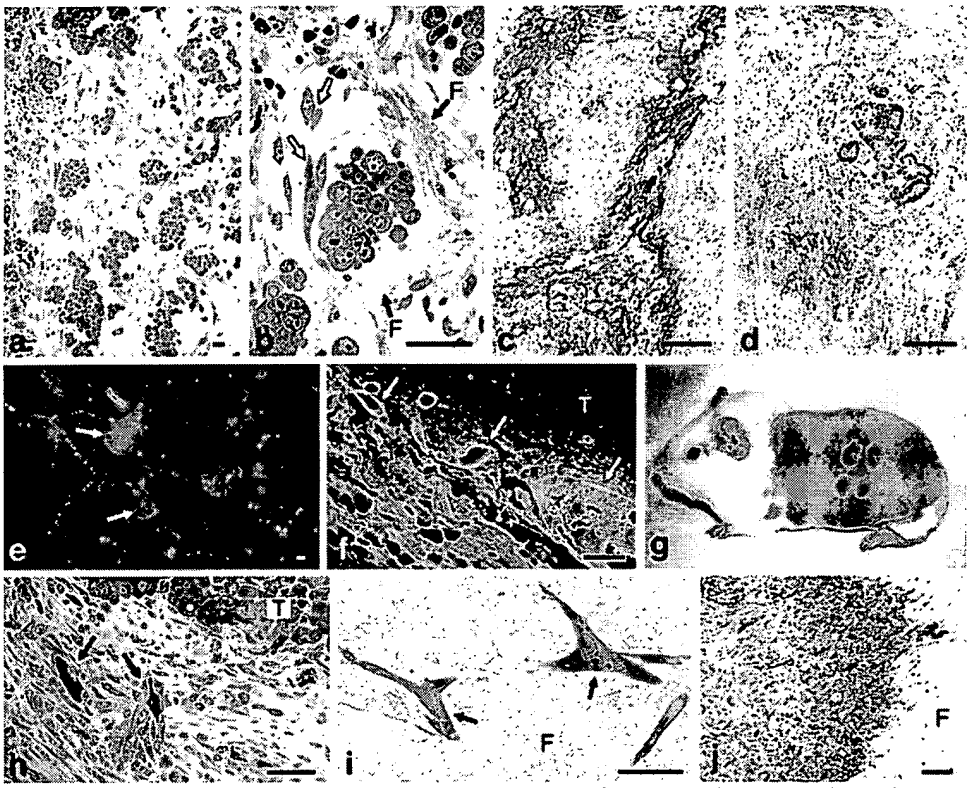

Figure 1. a and **b:** Line 10 tumor cells 48 hours after transplant into the subcutaneous space of syngeneic strain 2 guinea pigs. Fibrin forms a water-trapping gel (F) that serves as a provisional stroma that separates tumor cells into discrete islands and that provides a favorable matrix for fibroblast (**white arrows**) and endothelial cell migration. **c** and **d:** Immunohistochemical demonstration of fibrin (brown staining) in guinea pig line 1 and human colorectal adenocarcinoma, respectively. **e** and **f:** Blood vessels (**arrows**) supplying line 10 guinea pig tumors are hyperpermeable to circulating macromolecular fluoresceinated dextran. **g:** Miles assay illustrating permeability to Evans blue-albumin complex at sites of intradermal injections of the following: (**top**, left to right) neutralizing anti-VEGF-A antibody, line 10 guinea pig tumor ascites fluid, mix of line 10 tumor ascites fluid and control IgG, mix of tumor ascites fluid and neutralizing anti-VEGF-A antibody. **Bottom**: line 1 tumor ascites fluid, mix of line 1 tumor ascites fluid and control IgG, and mix of line 1 tumor ascites fluid and neutralizing anti-VEGF-A antibody. **h:** Fibroblasts and blood vessels (**black arrows**) invade line 1 tumor fibrin gel, replacing it with fibrous connective tissue. **i:** Fibroblasts (**arrows**) migrate through fibrin gel (F) *in vitro*. **j:** Implanted fibrin gel (F) in subcutaneous space is replaced by ingrowth of fibroblasts and new blood vessels, creating granulation-like vascular connective tissue. Scale bars, 25 μm (**b,i**); 50 μm (**a,c,d,h**); 100 μm (**e,f,j**). **e–g:** Reprinted[5] from *Curr Top Microbiol Immunol* 1999, 237:97–132 by copyright permission of Springer-Verlag GmbH & Co. KG; **j** reprinted[29] from *Lab Invest* 1987, 57:673–686 by permission of Lippincott Williams & Wilkins, copyright The United States and Canadian Academy of Pathology, Inc.

Hyperpermeable vessels were especially prominent at the tumor-host interface and it was therefore not certain from our studies whether tumor cells were permeabilizing normal host microvessels and/or were generating the formation of new, abnormal blood vessels that were intrinsically permeable. (Both of these possibilities were subsequently shown to be correct). To address the first possibility, we collected tumor cell culture supernatants and ascites fluids and found to our delight that both contained an activity that potently increased the permeability of microvessels in normal guinea pig skin (Figure 1g)✦ . We called this activity vascular permeability factor (VPF).

VPF was found to be non-dialyzable, largely inactivated by heat, and both cold and puromycin profoundly depressed its appearance in culture medium. Also, the vascular permeabilizing activity of tumor culture supernatants was not inhibited by antihistamines nor were these supernatants able to degranulate or release histamine from basophilic leukocytes or mast cells.[12] We concluded from these studies that the VPF activity present in tumor culture supernatants and ascites fluids was a secreted protein and one that increased vascular permeability by acting directly on blood vessel endothelium.

Using the Miles assay to screen column fractions for their ability to induce vascular permeability, Donald Senger then purified VPF to homogeneity.[13,14] Sometime later, our colleagues at the Monsanto Company[15,16] made use of Senger's N-terminal amino acid sequence data to clone VPF and show that it induced angiogenesis; independently, Napoleon Ferrara and his colleagues[17] at Genentech cloned VPF and reported that it was mitogenic for cultured endothelial cells. As a result of these studies, VPF has come to be known more widely as vascular endothelial growth factor (VEGF) and, more recently, as VEGF-A.

VEGF-A and Its Activities

Over the past two decades much has been learned about VEGF-A.[4,5,18] It is the founding member of a family of structurally related proteins with varying degrees of vasculogenic, angiogenic, and lymphangiogenic activity.[4,5,18,19,20] The VEGF-A gene is alternatively spliced to yield major isoforms of 189, 165, and 121 amino acids; the murine homologues are in each case one amino acid shorter. Of these, the 164/5 isoform is generally expressed most abundantly. However, all three isoforms are essential for normal development and inactivation of even a single copy of the VEGF-A gene results in embryonic death from failure of vascular development.[18,21,22] VEGF-$A^{164/5}$ is expressed by the vast majority of malignant animal and human tumors,[4,5] and interference with its activity, as by blocking antibodies or soluble receptors, inhibits tumor angiogenesis and tumor growth and may cause pre-existing tumors to regress.[18]

VEGF-$A^{164/5}$ is a multifunctional cytokine with a vascular permeabilizing activity some 50,000 times more potent than histamine. It also stimulates endothelial cell migration, is mitogenic for endothelial cells and is active in standard angiogenesis assays. Important for these activities is its ability to reprogram endothelial cell gene expression, leading to the up-regulation of many proteins including various proteases, tissue factor, a glucose transporter, etc. VEGF-$A^{164/5}$ is also an endothelial cell survival factor, able to avert both endothelial cell apoptosis and senescence.[23,24] As far as is known, all of the activities of VEGF-$A^{164/5}$ are mediated through two receptor tyrosine kinases (VEGFR-1, flt-1) and VEGFR-2 (flk-1, KDR), and through a more recently described, non-tyrosine kinase receptor, neuropilin.[19,25]

Expression of VEGF-A^{164} in Vivo

To investigate its capacity to induce tumor-like blood vessels and stroma, we sought to express VEGF-A^{164} in normal mouse tissues at a constant rate for a sufficient period of time to induce angiogenesis and new stroma. To that end we made use of a non-replicating adenoviral expression vector.[26] Such vectors are readily engineered and offer a number of advantages for investigating the potential functions of angiogenic cytokines. 1) They can be injected in any accessible tissue of adult or embryonic mice where they direct infected host cells to express gene inserts. 2) Protein expression levels

are readily adjusted by varying the dose of injected virus. 3) Control vectors engineered to express non-inflammatory proteins give insignificant backgrounds. 4) Combinations of cytokines can be studied, together or in sequence, by injecting vectors engineered to express different genes. 5) Adenoviral vectors are not integrated into the host cell genome and so the genes they introduce are not expressed indefinitely; this was actually an advantage for our purposes because it permitted us to study the effects of cytokine withdrawal on newly formed blood vessels and stroma.

The Vascular Response to VEGF-A^{164} Expression

Within hours of injection into any of several different tissues of nude mice or rats, infected host cells began to express VEGF-A^{164} mRNA and continued to do so at steady levels for 10 to 14 days, generating a strong angiogenic response (Figure 2)✦ ;[26] thereafter, VEGF-A^{164} expression declined gradually and, by 4 to 6 weeks was no longer detectable by *in situ* hybridization.

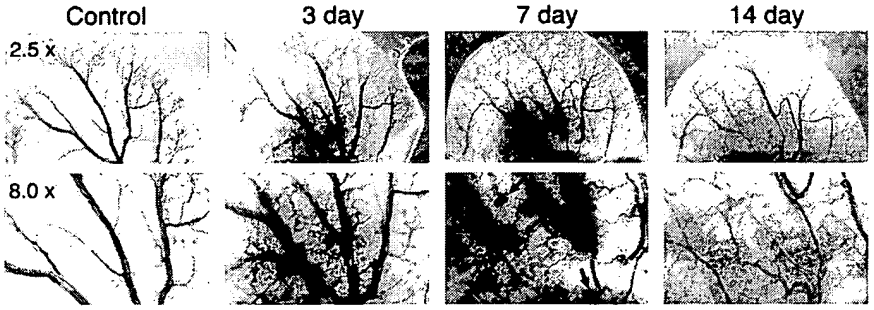

Figure 2. Angiogenic response to Ad-VEGF-A^{164} in the ears of nude mice at the indicated times and magnifications. (Reprinted[20] from *Cold Spring Harbor Symp Quant Biol* 2002, 67:227–237 by copyright permission of Cold Spring Harbor Laboratory Press.)

The Initial Response to Ad-VEGF-A^{164}: Fibrin Deposition and Edema

The initial response (1 to 3 days) was similar in all tissues studied and consisted of vascular hyperpermeability, fibrin deposition, and edema.[26] Generation of an extravascular fibrin gel requires not only leaky blood vessels but also activation of the clotting system. Clotting is triggered in most tissues within minutes of plasma leakage by tissue factor, a procoagulant expressed on tumor cells and many normal tissue cells.[27,28] Tissue factor reacts with clotting factor VIIa and subsequently with other extravasated plasma-clotting proteins, culminating in thrombin generation, clotting of fibrinogen to fibrin, and subsequent cross-linking of fibrin by activated factor XIII. Once deposited, cross-linked fibrin behaves as a gel that causes edema by trapping extravasated plasma and provides a proangiogenic provisional stroma similar to that observed in transplanted tumors and healing wounds.[1,11,12,29]

The fibrin deposited in tissues by tumors or by Ad-VEGF-A^{164} represents a net balance, at any moment of time, between fibrinogen influx, clotting and fibrinolysis. Fibrinogen influx results from leakage of plasma from permeabilized vessels and clotting from activation of the tissue factor pathway. However, the plasma protein plasminogen also extravasates from leaky blood vessels where plasminogen activators cleave it to generate plasmin, a potent fibrinolytic protease. VEGF-A^{164} selectively up-regulates plasminogen activator activity in endothelial cells, and tumors commonly express this enzyme so that deposited fibrin is continually being remodeled.[30]

VVOs: The Principle Pathway for Macromolecule Extravasation from Hyperpermeable Blood Vessels

To investigate the anatomical pathway(s) by which macromolecules extravasated from Ad-VEGF-A^{164} permeabilized vessels, we injected anionic ferritin intravenously as a tracer. Ferritin is an iron-rich plasma protein that can be visualized directly by electron microscopy. In agreement with earlier studies of tumor vessels[31] and normal skin following local injection of VEGF-A^{165} protein,[32,33] ferritin

extravasated from venules by a trans-endothelial cell pathway, that provided by the vesiculo-vacuolar organelle (VVO). VVOs are grape-like clusters of uncoated, largely para-junctional, cytoplasmic vesicles and vacuoles that traverse endothelial cytoplasm from lumen to ablumen (Figure 3)✦ .[31-35] The individual vesicles and vacuoles that comprise VVOs are linked to each other, and to the luminal and abluminal plasma membranes, by stomata that are normally closed by thin diaphragms. By some as yet unknown mechanism, VEGF-$A^{164/5}$ (and other vasoactive mediators) causes these stomata to open, providing a pathway for plasma and plasma protein extravasation. By contrast, interendothelial cell junctions remained tightly closed and did not admit tracer ferritin, even in the presence of abundant VEGF-$A^{164/5}$.

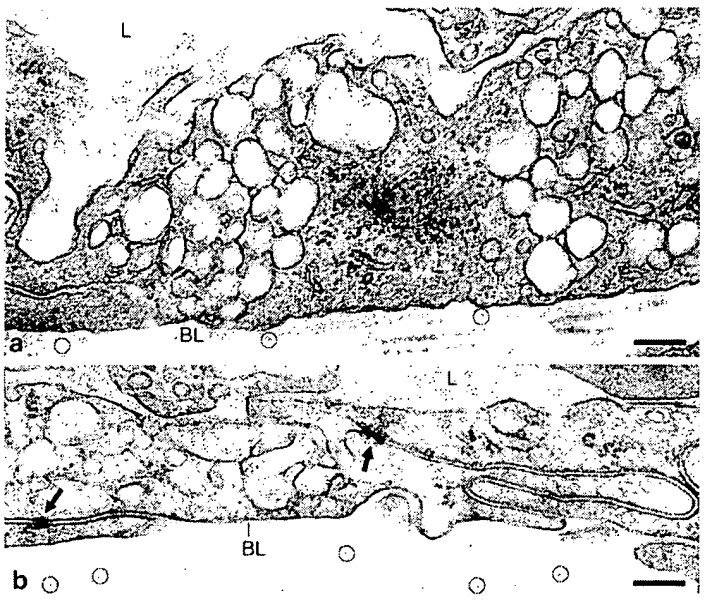

Figure 3. Electron micrographs 3 days after local Ad-VEGF-A^{164} injection and 20 minutes after i.v. injection of ferritin tracer. **a:** Hyperpermeable venule lined by endothelial cells of normal height, illustrating two prominent VVOs (collections of vesicles/vacuoles) that span endothelial cell cytoplasm from lumen (L) to abluminal basal lamina (BL). Note ferritin particles in vascular lumen, in VVO vesicles and vacuoles, and beneath BL (**black dots, encircled**). **b:** Mother vessel with greatly thinned endothelium spanned by four or fewer VVO vesicles/vacuoles. Note ferritin in these vesicles and vacuoles but tight junctions (**arrows**) remain closed and do not admit tracer. Bars, 200 nm. (Reprinted[26] from *Lab Invest* 2000, 80:99–115 by permission of Lippincott Williams & Wilkins, copyright The United States and Canadian Academy of Pathology, Inc.)

Formation of "Mother" Vessels from Pre-Existing Venules

Within 18 hours of Ad-VEGF-A^{164} injection, enlarged, thin-walled, serpentine, pericyte-poor, hyperpermeable, and strongly VEGFR-2-positive sinusoids appeared, structures to which we have given the name "mother" vessels (Figure 4)✦ . Vessels of this description are commonly found in human and animal tumors (Figure 4k)✦ [35] and are also transiently present in healing wounds (Figure 4l)✦ .[36] They continued to develop for ~5 days following injection of Ad-VEGF-A^{164} into tissues.

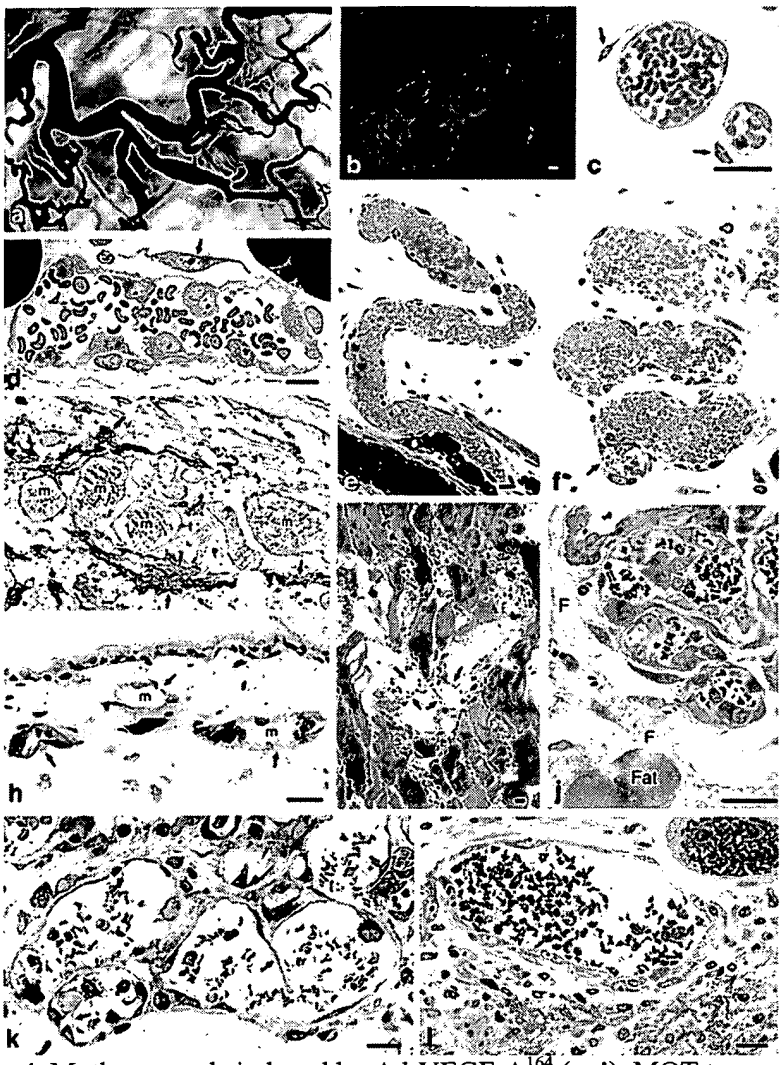

Figure 4. Mother vessels induced by Ad-VEGF-A^{164} (**a–j**), MOT tumor (**k**) and healing skin wound (**l**). **a:** Whole mount of colloidal carbon-perfused vascular bed. Mother vessels appear as enlarged segments of much smaller, normal venules. **b:** Confocal microscopic image of mother vessel stained for pericytes with an antibody to α-smooth-muscle actin. Note incomplete pericyte covering, especially over segments of greatest vessel enlargement. **c:** Developing mother vessels illustrating pericyte detachment (**arrows**). **d:** Mother vessel with detached pericyte (**arrow**) and activated endothelial cells whose large nuclei bulge into the vascular lumen, creating a highly irregular surface. **e** and **f:** Serpentine mother vessels with irregular luminal surface and endothelial cell bridging (**f, arrow**). **g:** Mother vessels (m) embedded in fibrin (**arrows**) provisional stroma. **h:** Loss of laminin staining (**arrows**) in developing mother vessels (m). **i** and **j:** Endothelial cell bridging (**i, arrows**) in mother vessels in skeletal muscle and ear skin, respectively. **k** and **l:** Mother vessels in mouse ovarian tumor and healing rat skin wound, respectively. Note bridging in **k** (**arrows**) and fibrin in **l** (lower, between cells). Scale bars, 20 μm (**b–l**); 100 μm (**a**). **a** and **b:** Reprinted[20] from *Cold Spring Harbor Symp Quant Biol* 2002, 67:227–237 by copyright permission of Cold Spring Harbor Laboratory Press; **c,e,f,h–j** reprinted[26] from *Lab Invest* 2000, 80:99–115 by permission of Lippincott Williams & Wilkins, copyright The United States and Canadian Academy of Pathology, Inc.

Mother vessels arose from the enlargement of pre-existing microvessels, primarily venules. By 18 to 24 hours, mean cross-sectional microvessel area had increased by ~4-fold, mean vessel perimeter by ~

2-fold, and the percent of dermis occupied by microvessels by 4- to 7-fold.[26] Mother vessels formed by a multistage process that involved proteolytic digestion of vascular basement membranes, pericyte detachment, and spreading and thinning of endothelial cells to cover a greatly expanded surface area (Figure 3b and Figure 4)✦ . For the first 48 hours, mother vessels formed without significant endothelial cell or pericyte division; thereafter, both types of cells proliferated extensively.

The vascular basement membrane is an especially important impediment to microvessel enlargement. Intact basement membranes are non-compliant (inelastic) structures that limit vascular enlargement to an ~30% increase in cross-sectional area.[37] Therefore, structural changes in the vascular basement membrane were necessary to accommodate the dramatic increases in microvessel area that were associated with VEGF-A^{164}-induced mother vessel formation. VEGF-A is known to up-regulate the expression of endothelial cell proteases that degrade vascular basement membranes[4,18] and reduced or absent staining for laminin and other basement membrane components, indicative of basement membrane degradation, was demonstrated by immunohistochemistry in developing mother vessels (Figure 4h)✦ .[26]

A Role for VVOs in Mother Vessel Formation

In addition to providing a pathway for macromolecule extravasation, VVOs also had a critical role in mother vessel formation. They provided an intracellular store of membrane that could be rapidly mobilized to the plasma membrane to increase cell surface area and permit vessel enlargement. Normal venular endothelium stores an amount of membrane in VVOs that is equivalent to 2.25 times that of plasma membrane.[20] Therefore, transfer of VVO membranes to the cell surface has the potential to provide a >3-fold increase in endothelial cell perimeter without new membrane synthesis. Consistent with this possibility, mother vessel formation was accompanied by progressive endothelial cell thinning and reduction in VVO vesicles and vacuoles (Figure 3b✦ , unpublished data). Our findings, therefore, indicate that the endothelial cell spreading and thinning that accompany mother vessel formation can be accommodated, at least initially, by transfer of intracellular VVO membrane to the cell surface.

Mother Vessel Evolution

Mother vessels are transitional forms that evolved along several different pathways (Figure 5)✦ .[26] Many underwent a process of bridging, in which cells expressing endothelial markers projected processes into and across mother vessel lumens (Figure 4, f, i, j)✦ . It remains to be determined whether these cells represent locally-activated vascular endothelium or were instead derived from circulating bone marrow precursors.[38,39] Whatever their source, they formed transluminal bridges that divided blood flow into multiple smaller-sized channels. Similar bridging takes place in tumor mother vessels (Figure 4k)✦ , and we have postulated that at least some of the smaller channels separated from each other to form individual smaller-caliber daughter capillaries.[35,40]

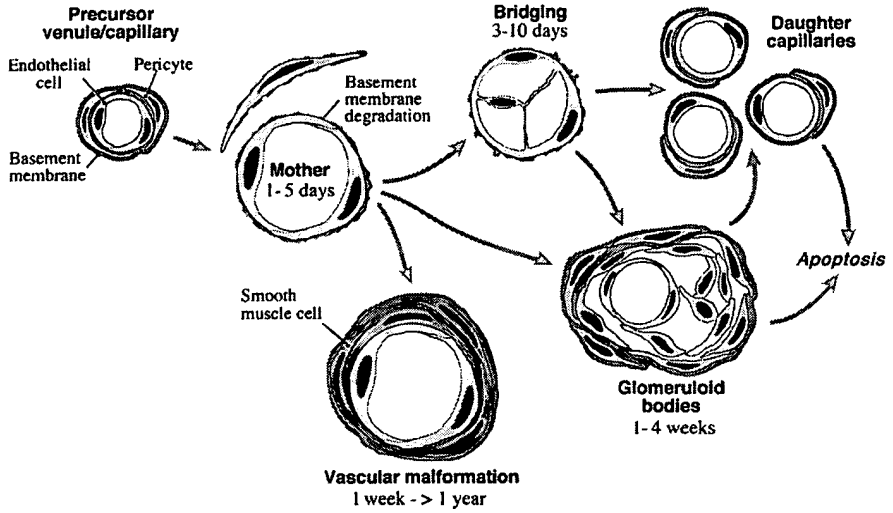

Figure 5. Schematic diagram of mother vessel formation and evolution into daughter capillaries, vascular malformations and glomeruloid bodies. (Modified[26] from *Lab Invest* 2000, 80:99–115 by permission of Lippincott Williams & Wilkins, copyright The United States and Canadian Academy of Pathology, Inc.)

Glomeruloid Bodies (GB)

GB are poorly organized vascular structures that resemble renal glomeruli (hence the name) and are a common feature of several human tumors, among them glioblastoma multiforme.[41] GB precursors were first recognized at ~3 days as focal collections of large, primitive cells in the endothelial lining of mother vessels (Figures 5 and 6)[42]. These cells bore endothelial cell markers, proliferated rapidly, and extended both into the vascular lumen and outwards into the extravascular connective tissue. By 7 to 10 days, pericytes were prominently included and organized themselves around endothelial cells to form primitive microvessels. As they expanded, GB severely compromised the mother vessels in which they had arisen, reducing and dividing originally large single vascular lumens into multiple, smaller, tortuous channels.

After about 14 days, as VEGF-A^{164} expression declined, GB progressively devolved by a process that involved both apoptosis and reorganization of endothelial cells and pericytes to form normal appearing capillaries (Figure 6i). By ~8 weeks, VEGF-A^{164} expression was no longer detectable and the angiogenic response had largely resolved with a return of microvascular density to near-normal levels. Taken together, these experiments provided the first animal model for generating GB, offered important insights into the mechanisms of GB formation and devolution, and demonstrated that VEGF-A^{164} is both sufficient for GB generation and necessary for their maintenance.

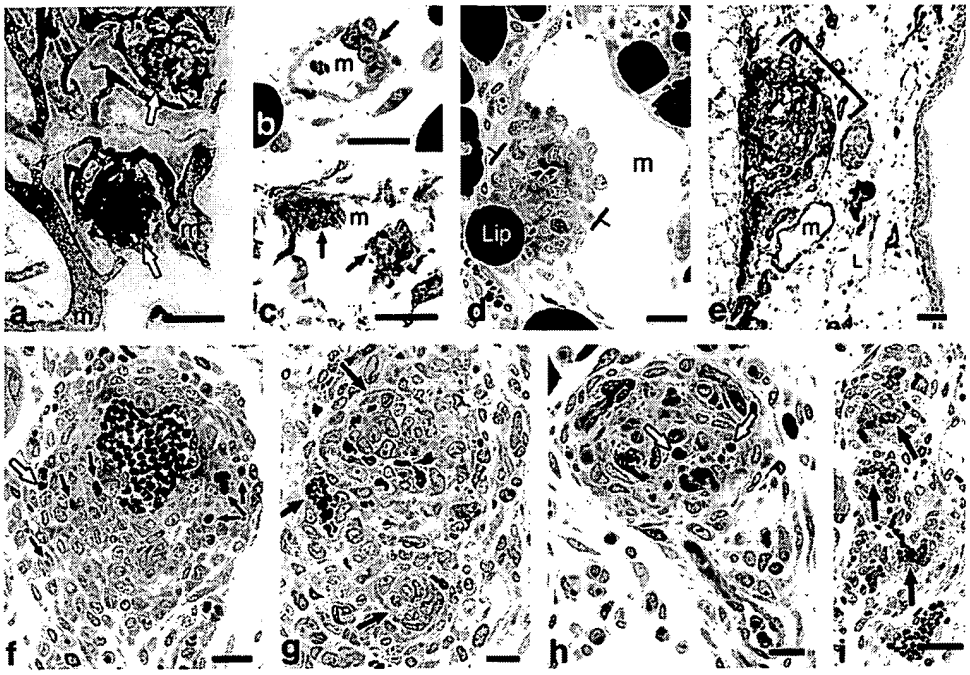

Figure 6. GB formation and devolution. **a:** Whole mount illustrates two GBs in ear of mouse perfused with colloidal carbon (**white arrows**), supplied by afferent and efferent mother vessels (m). **b:** 1-μm Epon section illustrates focal accumulation of large primitive cells (**arrow**) in a developing mother vessel (m). **c:** Immunohistochemistry demonstrates that such cells are CD31-positive. **d:** Primitive GB develops as focal nodule (between brackets) of proliferating cells in the wall of a mother vessel (m), extending both into the lumen and out into the extravascular connective tissue. Lip, adipocyte. **e:** Immunohistochemical staining for entactin demonstrates abundant basement membrane in a large GB (bracket) as well as in a mother vessel (m) and, conversely, very little in a lymphatic (L). **f** and **g:** Maturing GBs divide mother vessels into multiple, much smaller vascular channels (**arrows**). White **arrows** in **f** and **h** indicate apoptotic bodies. **h** and **i:** Devolving GBs reorganize into more normal-appearing microvessels (**black arrows**). Scale bars, 25 μm (**c–f, h–i**); 50 μm (**a,b**); 100 μm (**g**). (Reprinted[42] from *Am J Pathol* 2001, 158:1145–1160 by copyright permission of the American Society for Investigative Pathology.)

Vascular Malformations and Arteriogenesis

Thin-walled mother vessels lacking pericyte and basement membrane support are subject to thrombosis or collapse. While many mother vessels avoid this fate by evolving into smaller daughter vessels or GB, others maintained their large size by acquiring a coat of smooth muscle cells and/or collagen-expressing fibroblasts.[25] Such vessels were readily distinguished from normal arteries and veins by their inappropriately large size, by their thinner and often asymmetric muscular coat, and by the presence of perivascular fibrosis (Figure 7, a and b)+ . Vessels of this description closely resemble those found in vascular malformations.[43] This finding is of interest because it provides an animal model for generating vascular malformations and suggests that the vascular malformations observed in patients may be induced by VEGF-A overexpression. Once formed, these malformations persisted indefinitely (for more than a year), long after VEGF-A^{164} expression had ceased, indicating that they had achieved VEGF-A^{164} independence. In parallel with the formation of vascular malformations, small arteries and arterioles also responded to VEGF-A^{164} with modest replication of endothelial and smooth muscle cells, leading to the formation of larger, well-differentiated arteries (Figure 7, c–f)+ .[20]

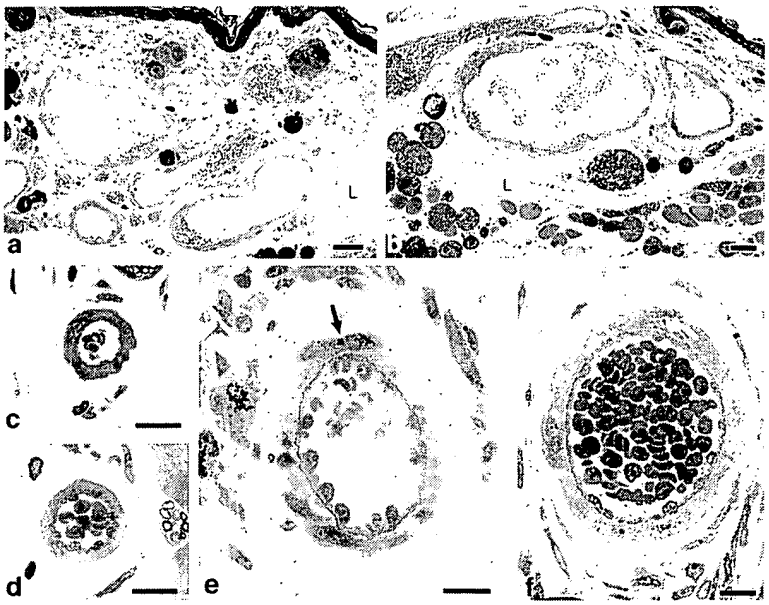

Figure 7. a and **b:** Vascular malformations induced by Ad-VEGF-A^{164} in ear skin at 35 and 131 days, respectively. L, giant lymphatics. **c–f:** Arteriogenesis following administration of Ad-VEGF-A^{164} in ear skin. Small arteries (**c**,**d**) enlarge by 6 to 7 days (**e**,**f**) accompanied by replication of endothelium and smooth muscle cells. **Arrow** (**e**), [^{3}H]thymidine incorporated by a smooth-muscle cell. Scale bars, 50 μm (**a**,**b**); 15 μm (**c–f**). (Modified[20] from *Cold Spring Harbor Symp Quant Biol* 2002, 67:227–237 by copyright permission of Cold Spring Harbor Laboratory Press.)

Lymphangiogenesis

Unexpectedly, VEGF-A^{164} also stimulated the proliferation of lymphatic endothelium, leading to the formation of a dense meshwork of tortuous, irregularly shaped, giant lymphatic channels (Figure 8)[44] These giant lymphatics were functional but poorly so, clearing tracers over a period of many hours rather than in a few minutes as in normal lymphatics.

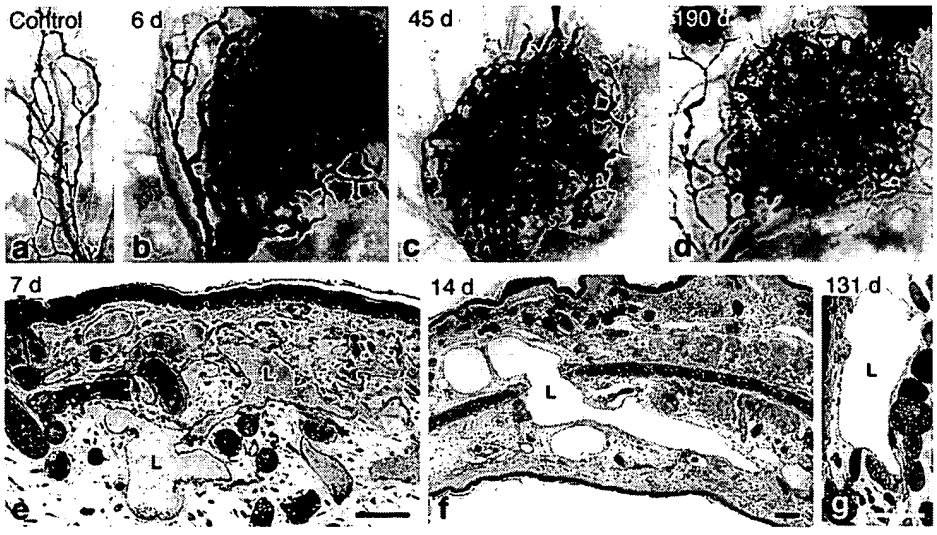

Figure 8. a–d: Giant lymphatics in mouse ear skin at indicated times after injection of Ad-VEGF-A^{164}.

Lymphatics were perfused with colloidal carbon and viewed macroscopically. **e–g:** 1-μm Epon sections of giant lymphatics at indicated times after Ad-VEGF-A^{164}. Lymphatics were injected with colloidal carbon (**e**). Scale bars, 50 μm. (Reprinted[44] form *J Exp Med* 2002, 196:1497–1506 by copyright permission of The Rockefeller University Press.)

Lymphangiogenesis developed in parallel with angiogenesis but at a slower pace. Enlarged lymphatics were first recognized at ~3 days after injection of Ad-VEGF-A^{164}, increased in size and number over the course of several weeks, and persisted indefinitely, in some instances for >1 year. Unlike venules, normal lymphatics lack pericytes and a well-developed basement membrane and are able to enlarge in response to edema.[45,46] Therefore, formation of giant lymphatics was largely attributable to replication of lymphatic endothelial cells without the need for basement membrane degradation, pericyte detachment, or endothelial cell thinning as was necessary for the formation of mother vessels.

Transformation of Provisional Fibrin Gel into Mature Connective Tissue Stroma

As was noted earlier, fibrin deposited in tumors and wounds imposes structure (Figure 1, a and b)✦ . It also provides an environment that supports cell migration (Figure 1, h–j)✦ . Endothelial cells, fibroblasts, and inflammatory cells express adhesion molecules (integrins) whose interaction with fibrin and fibronectin allows them to move freely in tumor and wound stroma.[29,47] As a consequence, fibrin facilitates both angiogenesis and the inward migration of fibroblasts. Over time, as VEGF-A^{164} expression declined, vascular permeability returned to normally low levels and the balance shifted from fibrin accumulation to fibrinolysis. Eventually, fibrin was digested, small blood vessels underwent apoptosis, and fibroblasts synthesized collagen, resulting finally in dense, relatively avascular connective tissue.[1,48]

Commentary

A number of conclusions can be drawn from these studies. First, VEGF-A^{164} sets in motion a complicated chain of events that leads to the generation of a vascular and connective tissue stroma that closely resembles that found in animal and human tumors. Because nearly all malignant tumors express VEGF-A$^{164/5}$, there is every reason to believe that tumors use this cytokine to generate stroma. Further, the steps and mechanisms by which tumors generate stroma closely mimic the events of wound healing in which VEGF-A$^{164/5}$ is also expressed.[36] Both tumor stroma generation and wound healing are characterized by vascular hyperpermeability, leakage of plasma, and clotting of extravasated fibrinogen to form a fibrin gel that serves as a provisional stroma for blood vessel and fibroblast migration. In both, the fibrin gel is degraded and the vascular connective tissue subsequently replaced by desmoplasia (tumors) or scar (wounds). However, whereas in healing wounds VEGF-A expression ceases with restoration of tissue normoxia, in tumors VEGF-A is expressed indefinitely. Thus, tumors may be likened to wounds that do not heal.[1]

Another important point is that the new blood vessels induced by Ad-VEGF-A^{164} and by tumors are not of a single kind but rather can be classified into at least several morphologically distinct types, ie, mother vessels, glomeruloid bodies, and vascular malformations. This heterogeneity may be expected to complicate anti-angiogenic and anti-vascular tumor therapy because not one but several distinct types of blood vessels must be targeted. Also, whereas mother vessels and glomeruloid bodies required continued VEGF-A^{164} expression for maintenance, vascular malformations did not and therefore would not be expected to respond to therapies that neutralized VEGF-A^{164} or its receptors. Consistent with this line of thinking, Benjamin et al[23] have shown that withdrawal of VEGF-A leads to the selective apoptosis of only those tumor microvessels that lack a pericyte coating.

In addition to inducing angiogenesis (formation of small blood vessels) VEGF-A^{164} also induced vascular malformations and arteries. These large vessels could have clinical significance quite apart from cancer. Administration of VEGF-A$^{164/5}$ and other angiogenic cytokines has been proposed as a novel approach for the treatment of coronary heart disease and other examples of tissue ischemia.[49] However, the angiogenesis induced by VEGF-A$^{164/5}$ would not be expected to offer much clinical benefit, first because the response is transient and second because the small vessels that form in response to VEGF-A

supply a newly induced stroma, not the native tissue. Instead, relief of tissue ischemia requires large patent arterial blood vessels that are located proximal to, not within, the ischemic tissue. The vascular malformations and arteries induced by VEGF-A^{164} might satisfy this criterion because they are of appropriate size and persist indefinitely, independent of VEGF-A. It is possible, therefore, that some of the clinical benefits that have been reported for gene therapy with VEGF-A[49] resulted not from angiogenesis but from unrecognized formation of these larger vessels.

The finding that VEGF-A induced lymphangiogenesis was unanticipated. Other members of the VEGF-A family, VEGF-C and VEGF-D, have been thought to be responsible for lymphatic development.[50] However, we found no evidence for VEGF-C or VEGF-D expression in our studies of Ad-VEGF-A^{164}, and it seems more likely that VEGF-A^{164} acted directly on lymphatic endothelial cells through VEGFR-2.[51] Our findings predict that malignant tumors that express large amounts of VEGF-A$^{164/5}$ would also induce the formation of abnormal lymphatics. Tumors have not been thought to generate new lymphatics[52] but this issue needs to be reinvestigated in light of our findings.

In summary, our work has demonstrated that a single cytokine, VEGF-A^{164}, induces remarkably diverse vascular responses and can account for at least three of the distinct types of abnormal blood vessels found in tumors. We do not yet know the secondary factors that are responsible for generating each of these vessel types and the larger number of such factors that must be needed to form the diverse types of mature blood vessels that are found in normal adult tissues. Discovering these secondary factors and elucidating their mode of action is an important future goal that will have important implications for understanding normal vascular development and for developing both pro- and anti-angiogenic therapies that have clinical utility.

Footnotes

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References

1. Dvorak HF: Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing. N Engl J Med 1986, 315:1650-1659[Medline]

2. Dvorak H, Nagy J, Feng D, Dvorak A: Tumor architecture and targeted delivery. Abrams P Fritzberg A eds. Radioimmunotherapy of Cancer. 2000:pp 107-135 Marcel Dekker, Inc., New York

3. Folkman J: Tumor angiogenesis. Adv Cancer Res 1985, 43:175-203[Medline]

4. Brown LF, Detmar M, Claffey K, Nagy JA, Feng D, Dvorak AM, Dvorak HF: Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. Exs 1997, 79:233-269[Medline]

5. Dvorak HF, Nagy JA, Feng D, Brown LF, Dvorak AM: Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. Curr Top Microbiol Immunol 1999, 237:97-132[Medline]

6. Warren BA: The vascular morphology of tumors. Peterson H-I eds. Tumor Blood Circulation: Angiogenesis, Vascular Morphology and Blood Flow of Experimental and Human Tumors. 1979:pp 1-47 CRC Press, Inc., Boca Raton, FL

7. Dvorak HF, Nagy JA, Dvorak AM: Structure of solid tumors and their vasculature: implications for therapy with monoclonal antibodies. Cancer Cells 1991, 3:77-85[Medline]
8. Beck L, Jr, D'Amore PA: Vascular development: cellular and molecular regulation. EMBO J 1997, 11:365-373
9. Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000, 6:389-395[Medline]
10. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J: Vascular-specific growth factors and blood vessel formation. Nature 2000, 407:242-248[Medline]
11. Dvorak HF, Dvorak AM, Manseau EJ, Wiberg L, Churchill WH: Fibrin gel investment associated with line 1 and line 10 solid tumor growth, angiogenesis, and fibroplasia in guinea pigs: role of cellular immunity, myofibroblasts, microvascular damage, and infarction in line 1 tumor regression. J Natl Cancer Inst 1979, 62:1459-1472[Medline]
12. Dvorak HF, Orenstein NS, Carvalho AC, Churchill WH, Dvorak AM, Galli SJ, Feder J, Bitzer AM, Rypysc J, Giovinco P: Induction of a fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. J Immunol 1979, 122:166-174[Medline]
13. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983, 219:983-985[Medline]
14. Senger DR, Perruzzi CA, Feder J, Dvorak HF: A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res 1986, 46:5629-5632[Abstract]
15. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT: Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989, 246:1309-1312[Medline]
16. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J: Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 1989, 84:1470-1478[Medline]
17. Leung DW, Cachianes G, Kuang W-J, Goeddel DV, Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989, 246:1306-1309[Medline]
18. Ferrara N, Keyt B: Vascular endothelial growth factor: basic biology and clinical implications. Exs 1997, 79:209-232[Medline]
19. Veikkola T, Alitalo K: VEGFs, receptors and angiogenesis. Semin Cancer Biol 1999, 9:211-220[Medline]
20. Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Manseau EJ, Dvorak AM, Dvorak HF: VEGF-A induces angiogenesis, arteriogenesis, lymphangiogenesis and vascular malformations. Cold Spring Harbor Symp Quant Biol 2002, 67:227-237[Medline]
21. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A: Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996, 380:435-439[Medline]
22. Carmeliet P, Ng YS, Nuyens D, Theilmeier G, Brusselmans K, Cornelissen I, Ehler E, Kakkar VV, Stalmans I, Mattot V, Perriard JC, Dewerchin M, Flameng W, Nagy A, Lupu F, Moons L, Collen D, D'Amore PA, Shima DT: Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nat Med 1999, 5:495-502[Medline]
23. Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E: Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 1999, 103:159-165[Abstract/Free Full Text]
24. Watanabe Y, Lee SW, Detmar M, Ajioka I, Dvorak HF: Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) delays and induces escape from senescence in human dermal microvascular endothelial cells. Oncogene 1997, 14:2025-2032[Medline]
25. Miao HQ, Lee P, Lin H, Soker S, Klagsbrun M: Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. EMBO J 2000, 14:2532-2539

26. Pettersson A, Nagy JA, Brown LF, Sundberg C, Morgan E, Jungles S, Carter R, Krieger JE, Manseau EJ, Harvey VS, Eckelhoefer IA, Feng D, Dvorak AM, Mulligan RC, Dvorak HF: Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. Lab Invest 2000, 80:99-115[Medline]
27. Dvorak HF, Quay SC, Orenstein NS, Dvorak AM, Hahn P, Bitzer AM, Carvalho AC: Tumor shedding and coagulation. Science 1981, 212:923-924[Medline]
28. Dvorak HF, Senger DS, Dvorak AM, Harvey VS, McDonagh J: Regulation of extravascular coagulation by microvascular permeability. Science 1985, 227:1059-1061[Medline]
29. Dvorak HF, Harvey VS, Estrella P, Brown LF, McDonagh J, Dvorak AM: Fibrin-containing gels induce angiogenesis: implications for tumor stroma generation and wound healing. Lab Invest 1987, 57:673-686[Medline]
30. Dvorak HF, Harvey VS, McDonagh J: Quantitation of fibrinogen influx and fibrin deposition and turnover in line 1 and line 10 guinea pig carcinomas. Cancer Res 1984, 44:3348-3354[Abstract]
31. Kohn S, Nagy JA, Dvorak HF, Dvorak AM: Pathways of macromolecular tracer transport across venules and small veins: structural basis for the hyperpermeability of tumor blood vessels. Lab Invest 1992, 67:596-607[Medline]
32. Feng D, Nagy JA, Hipp J, Dvorak HF, Dvorak AM: Vesiculo-vacuolar organelles and the regulation of venule permeability to macromolecules by vascular permeability factor, histamine, and serotonin. J Exp Med 1996, 183:1981-1986[Abstract]
33. Feng D, Nagy JA, Pyne K, Hammel I, Dvorak HF, Dvorak AM: Pathways of macromolecular extravasation across microvascular endothelium in response to VPF/VEGF and other vasoactive mediators. Microcirculation 1999, 6:23-44[Medline]
34. Dvorak AM, Kohn S, Morgan ES, Fox P, Nagy JA, Dvorak HF: The vesiculo-vacuolar organelle (VVO): a distinct endothelial cell structure that provides a transcellular pathway for macromolecular extravasation. J Leukoc Biol 1996, 59:100-115[Abstract]
35. Feng D, Nagy JA, Dvorak AM, Dvorak HF: Different pathways of macromolecule extravasation from hyperpermeable tumor vessels. Microvasc Res 2000, 59:24-37[Medline]
36. Brown LF, Yeo KT, Berse B, Yeo TK, Senger DR, Dvorak HF, van de Water L: Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. J Exp Med 1992, 176:1375-1379[Abstract]
37. Swayne GT, Smaje LH: Dynamic compliance of single perfused frog mesenteric capillaries and rat venules: a filtration coefficient correction. Int J Microcirc Clin Exp 1989, 8:43-52[Medline]
38. Rafii S, Meeus S, Dias S, Hattori K, Heissig B, Shmelkov S, Rafii D, Lyden D: Contribution of marrow-derived progenitors to vascular and cardiac regeneration. Semin Cell Dev Biol 2002, 13:61-67[Medline]
39. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997, 275:964-967[Abstract/Free Full Text]
40. Nagy JA, Morgan ES, Herzberg KT, Manseau EJ, Dvorak AM, Dvorak HF: Pathogenesis of ascites tumor growth: angiogenesis, vascular remodeling, and stroma formation in the peritoneal lining. Cancer Res 1995, 55:376-385[Abstract]
41. Lantos P, VandenBerg S, Kleihues P: Tumors of the nervous system. Graham D Lantos P eds. Greenfield's Neuropathology. 1997:pp 583-879 Arnold, London
42. Sundberg C, Nagy JA, Brown LF, Feng D, Eckelhoefer IA, Manseau EJ, Dvorak AM, Dvorak HF: Glomeruloid microvascular proliferation follows adenoviral vascular permeability factor/vascular endothelial growth factor-164 gene delivery. Am J Pathol 2001, 158:1145-1160[Abstract/Free Full Text]
43. McKee P: Pathology of the skin with clinical correlations. 1996:pp 16.57-16.66 Mosby International, London
44. Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, Lawitts JA, Benjamin L, Tan X, Manseau EJ, Dvorak AM, Dvorak HF: Vascular permeability factor/vascular endothelial growth factor

induces lymphangiogenesis as well as angiogenesis. J Exp Med 2002, 196:1497-1506[Abstract/Free Full Text]
45. Leak LV, Schannahan A, Scully H, Daggett WM: Lymphatic vessels of the mammalian heart. Anat Rec 1978, 191:183-201[Medline]
46. Feng D, Nagy J, Dvorak H, Dvorak A: Ultrastructural studies define soluble macromolecular, particulate, and cellular transendothelial cell pathways in venules, lymphatic vessels, and tumor-associated microvessels in man and animals. Microsc Res Tech 2002, 57:289-326[Medline]
47. Brown LF, Lanir N, McDonagh J, Tognazzi K, Dvorak AM, Dvorak HF: Fibroblast migration in fibrin gel matrices. Am J Pathol 1993, 142:273-283[Abstract]
48. Dvorak HF, Form DM, Manseau EJ, Smith BD: Pathogenesis of desmoplasia: I: immunofluorescence identification and localization of some structural proteins of line 1 and line 10 guinea pig tumors and of healing wounds. J Natl Cancer Inst 1984, 73:1195-1205[Medline]
49. Losordo DW, Vale PR, Isner JM: Gene therapy for myocardial angiogenesis. Am Heart J 1999, 138:132-141
50. Oh SJ, Jeltsch MM, Birkenhager R, McCarthy JE, Weich HA, Christ B, Alitalo K, Wilting J: vEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. Dev Biol 1997, 188:96-109[Medline]
51. Feng D, Nagy JA, Brekken RA, Pettersson A, Manseau EJ, Pyne K, Mulligan R, Thorpe PE, Dvorak HF, Dvorak AM: Ultrastructural localization of the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) receptor-2 (FLK-1, KDR) in normal mouse kidney and in the hyperpermeable vessels induced by VPF/VEGF-expressing tumors and adenoviral vectors. J Histochem Cytochem 2000, 48:545-556[Abstract/Free Full Text]
52. Jain RK, Fenton BT: Intratumoral lymphatic vessels: a case of mistaken identity or malfunction? J Natl Cancer Inst 2002, 94:417-421[Free Full Text]